Fabrication and characterisation of Extracellular matrix based composite films for wound healing application

Thesis submitted in partial fulfillment of the requirements for the degree

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in
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By

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CERTIFICATE

This is to certify that the thesis entitled, "**Fabrication and characterisation of Extracellular matrix based composite films for wound healing application**" submitted by **Mr. Susanta Basuri** in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology & Medical Engineering with specialization in "Biomedical Engineering" at National Institute of Technology, Rourkela is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other university/institute for the award of any Degree.

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Abstract – Wound healing, occurs naturally upon inflammation following a programmed process such as hemostasis, inflammation, proliferation, and remodeling. But at times this sequence is not followed due to lack of sufficient oxygenation, nutrition, stress etc. thus causing improper or impaired wound healing. To suffice this need, implants such as scaffolds are prepared which mimics the extracellular matrix (ECM) thereby providing a proper environment for growth, maintenance and adherence of the cells present in it. In the present work, we propose ECM derived from porcine omentumto be used as a xenogeneic biomaterial for designing our scaffolds. For this study porcine omentum was decellularised following a series of processes including both physical and chemical method such as repeated freeze thawingand SDS washing of the tissues. Composite films of both Alginate-ECM and Chitosan –ECM in different compositions were prepared by solvent casting method. These films were further characterized by Light Transmission, Swelling, hemocompatibility, moisture absorption and water vapour permeability, X-ray diffraction (XRD) and Fourier Transform Infrared (FTIR) spectroscopy. Alginate films without ECM were found to be less thick than the chitosan films without ECM. Upon swelling the films differed in the hydrophilicity as uptake of water by chitosan films were more than alginate films. This accounts to the moisture retention capacity of films Apart from this since ECM is more hydrophilic than alginate and chitosan and addition of higher proportion of ECM in films imparts them more hydrophilicity. Alginate films were found to have more thickness, more hemocompitibility, more crystallinity and higher rate of moister absorption, lesser swelling ratio and more water vapour permeability than chitosan films

Keywords: Porcine omentum, Decellularization, Extracellular matrix, Chitosan, Alginate, Chronic wounds

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Chapter 1

INTRODUCTION

Chronic wounds are the wounds that does not heal or improve significantly within predictable amount of time. Chronic wounds occur due to insufficient blood supply, abnormal ECM deposition and because of the dry conditions occurring in the body [1]. Since these chronic wounds take a lot of time to heal and thus it causes physical and emotional stress in many patients, so it is necessary to find a proper treatment for this.

Chitosan is a Polymer that is acquired from the hard skeleton of shell fish, including crab, lobster, and shrimp. It is utilized to treat obesity, high cholesterol, and Crohn's infection. It is aexcellent biopolysaccharides [2].Chitosan is profoundly hydrophobic and is insoluble in water. It is dissolvable in hexafluoroisopropanol, hexafluoroacetone and chloroalcoholsin conjugation with water results of mineral acids [3]. It is suitable as a transporter for its high thickness, charge appropriation and discharge systems. Further, because of its crystalline, hydrophobic nature and dynamic –OH and $–NH_2$ groups, Chitosan is novelpractical bio-macromolecules that have the aggregate impacts as pharmaceutical excipients, the biocompatibilityand biodegradability [4].

Alginate is a commonly happening anionic polymer normally gotten from tan ocean growth, and has been widely explored and utilized for some biomedical application, because of its biocompatibility, gentle gelation by expansion of divalent cations [5]. Alginate hydrogels have been especially alluring in wound recuperating, pill conveyance, and tissue building applications [6]. Alginate wound dressings keep up a physiologically sodden microenvironment, minimize bacterial contamination at the injury site, and encourage wound recuperating. Drug particles, from little compound pills to macromolecular proteins, could be discharged from alginate gels in a controlled way [7]. Alginate dressings keep up a physiologically sodden microenvironment that pushes recuperating and the framing of granulation tissue. Alginates could be washed away with saline watering system, so evacuation of the dressing does not meddle with recuperating granulation tissue. Alginate dressings are extremely helpful for moderate to intensely exuding wounds [8].

The extracellular matrix (ECM) is the non-cell part introduce inside all tissues and organs, and gives vital physical platform for the cell constituents. ECM is made out of proteins and polysaccharides [9]. The ECM is made out of two fundamental classes of macromolecules:proteoglycans and sinewy proteins. The fundamental stringy ECM proteins are collagens, elastins, fibronectins and laminin [10]. All cells in strong tissues are encompassed by extracellular matrix. Both plants and creatures have ECM. The cell divider of plant cells is a sort of extracellular matrix[11]. ECM gives: rigidity for tendons,compressive quality for cartilage,hydraulic security for some sorts of cells,elasticity to the dividers of blood vessels . We use ECM based bandage for chronic wounds because it can replace of abnormal ECM and growth factor attached with it [12].

In this study we worked for the treatment of chronic wounds and fabricated ECM composite bandages. The ECM derived from the porcine omentum was composited with Chitosan and Alginate in various proportion and those were characterized by various methods.

Objective

- Fabrication and characterisation of Chitosan-ECM composite films for wound healing application
- Fabrication and characterisation of Alginate-ECM composite films for wound healing application

Chapter 2

REVIEW OF LITARETURE

Moustafa M.G. Fouda*, R. Wittke, D. Knittel, "Use of chitosan/polyamine biopolymers based cotton as a model system to prepare antimicrobial wound dressing"

The main aim of the study was to explore and compare the antibacterial properties of chitosan and linear polyvinyl amine, as a biopolymer, with the prepared dressing based cotton. These treated cotton were further characterized by monitoring the susceptibility of the amino groups created on the surface of the fabric. The produced dressing based cotton can be used as a model system to treat wounds, ulcers as well as diabetic ulcers. Chitosan is a biopolymer that has been known as being able to accelerate the healing of wound in human. Chitosan simulated the migration of polymorph nuclear (PMN) as well as mononuclear cells and accelerated the re-epithelization and normal skin regeneration. Chitosan have antibacterial activity against a broad spectrum. The binding of chitosan with DNA and inhibition of mRNA synthesis occurs via the penetration of chitosan into the nuclei of the microorganisms and interfering with the synthesis of mRNA and proteins. Chitosan also facilitates wound repair. According to the results in this work, the antimicrobiala ctivity of chitosan/polyvinyl amine system showed promising results[13].

Rupesh Gajanan Nawalakhe, et al "Development of Electrospun Iminochitosan for Improved Wound Healing Application"

To explore the properties of chitosan derivatives, nanofibrous iminochitosan was prepared by electrospinning technique. The solvent used for dissolving iminochitosan before being electrospun is trifluoroacetic acid (TFA). Apart from this several other parameters including polymer concentration, electric field and extrusion rate were also investigated. Good fiber

formation occurred within a range of 3%-8% of iminochitosan concentration. The electrospinning concentrations in the range of 1%-5% were studied for antibacterial testing. The results indicate that the nanofiber webs exhibit excellent antimicrobial behavior[14].

Ali Demir Sezer,1 Fatih Hatipoğlu, et al "Chitosan Film Containing Fucoidan as a Wound Dressing for Dermal Burn Healing: Preparation and In Vitro/In Vivo Evaluation"

This study was done to develop chitosan based films containing fucoidan and to investigate its suitability for the treatment of dermal burns on rabbits. The prepared films were tested on the basis of porosity, thickness, swelling tests, tensile strength, water vapor permeability and bio adhesion of the films. It was observed thathigher chitosan concentration significantly increased tensile strength of the films. Also more porous the films more would be its water uptake property. The swelling test accounted to the retention property of the films. Furthermore, dermal burn healing experiments using rabbit have shown that the application of fucoidan chitosan film onto an open burn wound induces significant wound contraction, and accelerates the wound closure and healing process. Thus, the fucoidan-chitosan film may be a promising new dressing for wound occlusion and tissue repairing[15].

Immanuel M. Sebastine and David J. Williams "The Role of Mechanical Stimulation in Engineering of Extracellular Matrix (ECM) "

Mechanotransduction is a complex phenomenon requiring the selective involvement of many different signaling pathways in response to mechanical stimuli .The critical component of the mechanotransduction process is the ECM and the initial responses to mechanical stimuli are recorded at the proximities of cell-ECM contacts. This review focuses on the study of these pathways involved in engineering the ECM. Since, cells respond to mechanical stimuli and regulate the metabolic functions via mechanotransduction and synthesize ECM, in-vitro studies of mechanotransduction using automated bioreactors that are capable of mimicking the physiological environment by applying different loads will help us to examine how mechanical loads influence intracellular signaling, pathway and their behavior on ECM. Although the experiments conducted in microscopic tissues have demonstrated a strong correlation between mechanical forces and changes in cell behaviors, better understanding of mechanotransduction will help us to apply appropriate mechanical stimulation on cells in scaffolds in vitro for the expression of a specific gene of interest or creation of particular constructs. Development of new tools or sensors to observe the changes in cells during mechanotransduction and toanalyses the genes, mRNA, and proteins expressed within the tissue construct will open newa venues of research[16].

Biji Balakrishnana, M. Mohanty et al "Evaluation of an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin"

In situ wound dressings are better and has more advantages than the preformed dressings as it offers comfort without wrinkling or fluting in the wound bed, ease of application and improved patient compliance. This paper describes an in situ based wound dressing applications using hydrogels and use of certain biomaterials like gelatin, oxidized alginate and borax. As we know that

periodate oxidized alginate rapidly cross-links proteins such as gelatin in the presence of borax to give in situ forming hydrogels that are both non-toxic and biodegradable. This composite matrix has the haemostatic effect of gelatin, the wound healing-promoting feature of alginate and the antiseptic property of borax which makes it a potential wound dressing material. From the study conducted, the hydrogel was found to have a fluid uptake of 90% of its weight which would prevent the wound bed from accumulation of exudates. The water vapor transmission rate (WVTR) of the hydrogel was found to be 26867124 g/m^2 day indicating that the hydrogel can maintain a moist environment over wound bed in moderate to heavily exuding wound which would enhance epithelial cell migration during the healing process. A rat model was used for demonstrating, the efficacy of hydrogel in wound healing and it was found that within 2 weeks, the wound covered with gel was completely filled with new epithelium without any significanta dverse reactions. These in situ forming hydrogels fulfil many critical elements desirable in a wound dressing material. Thus, this can act as a promising approach serving the purpose[17].

R. Jayakumar1, M. Prabaharan, P. T. et al, "Novel Chitin and Chitosan Materials in Wound Dressing"

As Skin plays an important role in homeostasis and the prevention of invasion by microorganisms, use of a biomaterial as a support material can help solve the issue. This review particularly, focuses on the affectivity of chitin and chitosan as wound dressing material and mechanisms of such action in the molecular, cellular, and systemic levels. Chitin and its derivative, chitosan, are biocompatible, biodegradable, nontoxic, anti-microbial and hydrating agents. Due to these properties, they show good biocompatibility and positive effects on wound Healing. Chitin is an abundant polysaccharide and chitosan is a deacetylated product of chitin. Both chitin and chitosan

has beneficial biological and antimicrobial properties and has potential for wound healing. Healing pattern gets altered in chronic wounds which lead to scars and unwanted tissue damage which might be a result of environmental irritants as well as water and electrolyte disturbances. The ordered regeneration of wounded tissues requires the use of chitin and chitosan in the form of non-woven, Nano fibrils, composites, films, scaffolds and sponges. Thus, these naturally occurring biomaterials can help restore the damaged tissue, thereby serving in the field of tissue engineering[18].

Chapter 3

MATERIALS AND METHODS

3.1 Decellularization and solubilisation of Adipose Tissue:

Pig omentum tissue were collected from the butcher house, Malegam, Rourkela, Orissa, India. Tissue were put away in sterile plastic bags and put away at 4ºC. The Clean parts of the collected Pig omentum washed with distilled water 3-4 times to evacuate all the blood. Cut into little pieces. This was then blended with twofold volume of refined water and blended for 5min utilizing a house hold blender[21]. Mix result put in 50 ml tube. After settling the mixed glue were divided into three layers: oil, ECM layer, and water. Oil layer was shaped because of the lysis of tissue which was at long last differentiated upon centrifugation at 6000 rpm for 15 min at room temperature. The center layer holding omentum and ECM were gathered tossing the upper oil layer and more level water rich layer. 5gm of the decellularized tissue was solubilized in 100 ml of 0.5m acidic corrosive for further transforming. The solubilized ECM were freeze dryer and put away at 4 ^{0}c for further use.

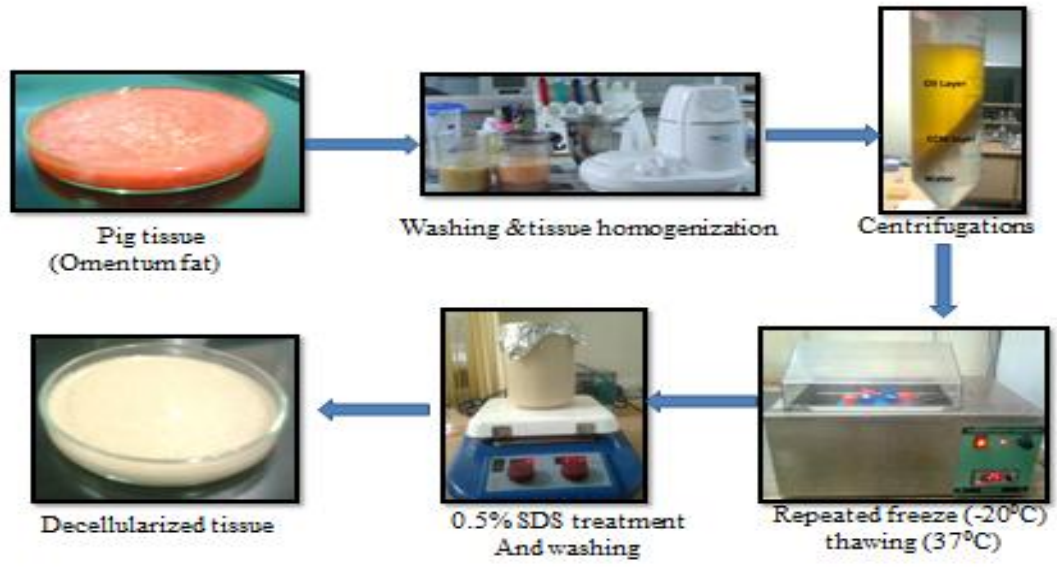


Fig 1: Decellularization of omentum

3.2 Preparation of Solutions:

3.2.1 Polymer solution preparation: For arrangement of polymer result, 2gm/dl of chitosan solution was mixing in 0.5 M acetic acid . Chitosan powder was gradually added to 0.5 M acetic acid solution under stirring condition for 24hrs until a homogenous solution was formed.

For preparation of Alginate solution, we take 2gm Sodium Alginate. It was slowly added to Distilled water for 1 day until a homogenous solution was formed.

3.2.2 ECM solution preparation:

For ECM solution, 5gm of ECM was dissolved in acetic acid solution .It was dissolved in 0.5 M acetic acid solution (4.5ml/150ml). Thus, ECM solution were prepared. Other hand another 150 mg/ml of ECM solution was prepared. In this time ECM was dissolved in NaOH solution.

3.2.3 Preparation of Composite Films:

Casting/ Solvent evaporation Technique was emulated to prepare the blend films in different ratios. Arranged Chitosan & ECM solutions were taken in 5:0, 4:1, 3:2, 2:3, 1:4, (w/w) ratios individually. The mixture was mixed until homogenous solutions was formed which was trailed by 4 hrs of degassing. The chitosan control specimen was additionally ready in the same path, aside from it had refined water rather than ECM. 30 gm of the arranged chitosan – ECM solutions were spilled in the little Petri plates (90 mm width) then dried for 24 hours in the vacuum oven at temperature of 37°c. After drying we get chitosan-ECM composite films.

For prepared Alginate and ECM solution also take in 5:0, 4:1,3:2,2:3,1:4 (w/w) ratios respectively. In case use ECM which was dissolved in NaOH solution.

3.4 Characterizations of ECM Films:

3.4.1 Thickness of the films:

Digital caliper (Traceable® Digital Calipers 6in, Fischer Scientific) was utilized to measure the thickness of the films at different portions of the film. The final thickness was calculated as mean ofthe estimations at 3 locations of the film.

3.4.2 Film Transparency/ Film Light Transmission Test:

A sample is placed in the UV & VIS light .The spectral transmittance is obtained by measuring the transmittance of 3 mm thick glass with film attached to one face[24]. For these tests, we determined the visible light transmittance, UV transmittance of film adhered to glass. The measurements were performed on light incident from the glass surface using a UV-VIS spectrophotometer.

Measuring Instrument	**UV-Spectrophotometer(Double BeamSpectrophotometer 2203)**
Measurement wavelength Range	**200 nm to 700nm**
Scan Speed	**Medium**
Film	**Chitosan-ECM Film, Chitosan-Alginate Film**

Table 1: Specifications of UV-Spectrophotometer

3.4.3 Swelling Test:

The Films(chitosan-ECM Films and Alginate-ECM Films) of 1x1 cm^2 size were taken in a 6 well plate. Dry weight of the films were noted. These films in the wake of peeling were weighed and drenched in PBS (pH 7.4) at room temperature. The films were expelled from PBS and smeared on filter paper to uproot approximately bound water and wet weights of the films (W t) were noted after customary interim of time (5, 10, 20, 30, 60, 120 min) separately[25]. The experiment was performed in triplicates and mean worth was taken. The swelling ratio (%) might be defined as the degree of the weight increment to the introductory dry weight films.

Swelling ratio **(%) = [(**Wet weight of films-Dry weight of films**)/**Dry weight of films**] %**

3.4.4 Moisture Test:

Moisture tests are due to check the amount of moisture absorb in materials when they were exposed to environmental condition. In our study we chose 84% of humidity and 30°C temperature keeping in view the atmosphere of India. The 84% humidity was created by supersaturated solution of Kcl inside the desiccator and the desiccator was kept outside environment. The filmswere weighed again after 24 hrs of drying and rate of water misfortune was computed which shows the dampness substance introduce at first in the film[26].

Percentage moisture absorbed (%) = **[(**Weight (final) of the films after drying – Initial weight of the films**)/**finalWeightof the films after drying**] %**

3.4.5 Water Vapor Transmission Permeability Test:

The property of films to penetrate is proportional to the surface geography of the film, which is controlled by Water vapor transmission test utilizing the water system. As indicated by this technique, the Chitosan-ECM films were kept totally stuck on to the highest point of a barrel shaped glass tubes utilizing a Teflon tape. At first filled with 30 ml PBS and the introductory weight and stature of the water was recorded and after that the tubes were kept at 37°c. The readings were taken in regular interval 6hrs, 12 hrs, 1 day, 2 days to 6 days. The transmission of water vapor was calculated[27].

$$\text{Water Vapor Transmission rate (g/day-m}^2\text{)} = (\text{Change in weight} \times \text{Test area}) \div \text{Time}$$

3.4.6 Hemocompatibility Test:

The hemocompatibility test were performed utilizing leachants of the ECM–chitosan Films and ECM-alginate Films. The film of 1x1 cm^2size were taken in a 12 well plate. The films drenched into 10 ml of PBS. pH value of PBS is 7.4. Then it kept in shaking incubator, under stirring rate of 60 rpm at 37°c for 10 minutes. For This test, fresh goat blood was taken. Than it diluted in 1:1 ratio with 0.9% normal saline. 0.5ml of leachants was included blood suspension took after by the sample incubation at 37°c for 1 hour. From that point, the samples were centrifuged at 4000 rpm and the supernatant was investigated for the optical thickness. For making the positive control, we add the 0.1N HCL. And for negative control we use normal saline. Than The % hemolysis was measured.

$$\%\ \text{Hemolysis} = [(\text{Test sample-Negative Control}) \div (\text{Positive Control} - \text{Negative Control})] \times 100$$

3.4.7 Fourier Transform Infrared Spectroscopy:

Fourier Transform Infrared Spectroscopy is a method which is used to obtain infrared spectrum of transmission, absorption of materials. FTIR is maybe the most compelling device for recognizing sorts of chemical bonds[28]. The wavelength of light absorbed is characteristic of the chemical bond.The chitosan-ECM composite films and alginate-ECM films were scanned for spectroscopic analysis using FTIR spectroscopy ATR mode. These specimens were examined keeping air as the reference. Reading of air was at first taken by the machine for foundation subtraction and afterward the samples were set in machine to record FTIR readings, accordingly subtracting the crests acquired via air.Scanning range was 4000 cm^{-1} to 500 $cm^{-1}$with a resolution of 4 cm^{-1}.

3.4.8 X- Ray Diffraction:

X-Ray Diffraction is a technique that is used to study for crystalline material. The 3D stricter of non-amorphous material is defined by fixed, repeating planes of atoms which form a crystalline lattice. When a X-ray beam interacts with planes of atoms, some part of the X-Ray beam is transmitted, some part is absorbed by the sample, some part is deflected and scattered[29]. When an X-ray hits a sample and is diffracted, we can measure the distances between the planes of the atoms that Present in the sample by using Bragg's Law.

$$\mathbf{n\,\lambda = 2d\sin\theta}$$

[Where, λ= wavelength of the incident X-Ray, n= order of the diffracted beam, d= distance between adjacent plane of the atoms]

The Chitosan – ECM and Alginate – ECM composite films were examined using X-ray diffractometer(PW3040, XRD – PANanalytical, Philips, Holland). Cu – Kα use as a radiation source.

Wavelength is 0.154 nm. It was worked at 30 KV and 20 mA. Scanning of the specimens was carried out at 5° - 50° .Scanning rate is 2° 2θ/min.

SI.no.	Chemical name	Catalogue no.	Company name
1.	Chitosan	Grm9358	Himedia Laboratories Pvt Ltd
2.	SodiumAlginate	40105 K05	SDFCL Limited
3.	Acetic acid	0000502500	LOBA CHEMIE
4.	NAOH	0589800500	LOBA CHEMIE
5.	Glycerol	G0010	RFCL LIMITED
6.	NACL	0581901000	LOBA CHEMIE
7.	KCL	13305	QUALIGENS
8.	NA2HPO4	18825	QUALIGENS
9.	KH2PO4	P0320	RFCL LIMITED

Table 2:Description of Materials used for Project work

Chapter 4

RESULTS & DISCUSSIONS

4.1Thickness Test:

The thickness of the films were measured using a Vernier calipers. Readings were taken from different portions of the films and the mean readings were noted.

Sl. No.	EDGE1(mm)	EDGE2(mm)	CENTER	AVG ± S.D.
1	0.04	0.06	0.08	0.06 ± 0.02
2	0.09	0.09	0.10	0.093 ± 0.005
3	0.08	0.09	0.08	0.083 ± 0.005
4	0.10	0.10	0.14	0.113 ± 0.023
5	0.09	0.09	0.11	0.103 ± 0.011

Table 3: Thickness test result of Chitosan- ECM Film

Sl. No.	EDGE1(mm)	EDGE2(mm)	CENTER	AVG ± S.D.
1	0.03	0.03	0.03	0.03 ± 0
2	0.36	0.29	0.23	0.293 ± 0.065
3	0.25	0.28	0.25	0.26 ± 0.017
4	0.23	0.27	0.25	0.25 ± 0.02
5	0.24	0.24	0.29	0.256 ± 0.028

Table 4: Thickness test result ofAlginate- ECM Film

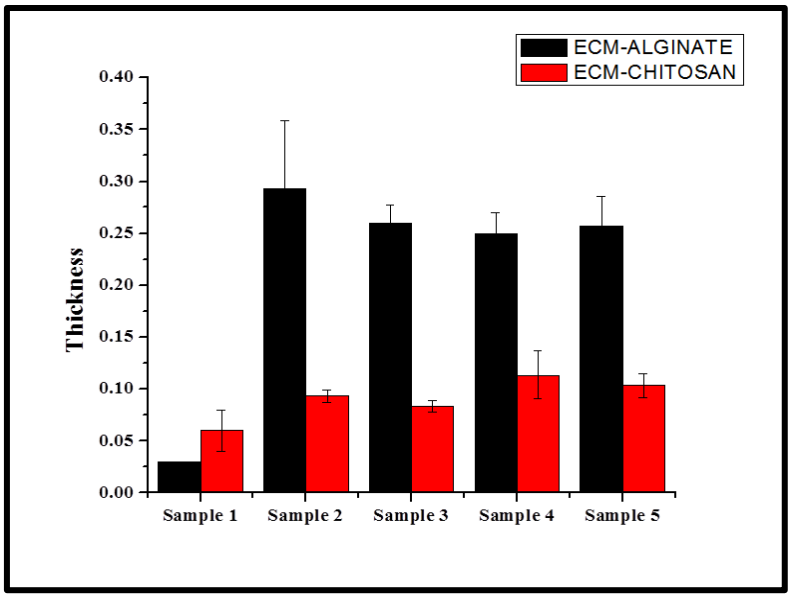


Fig 2: Film Light Thickness test ofChitosan-ECM Film & Alginate- ECM Film

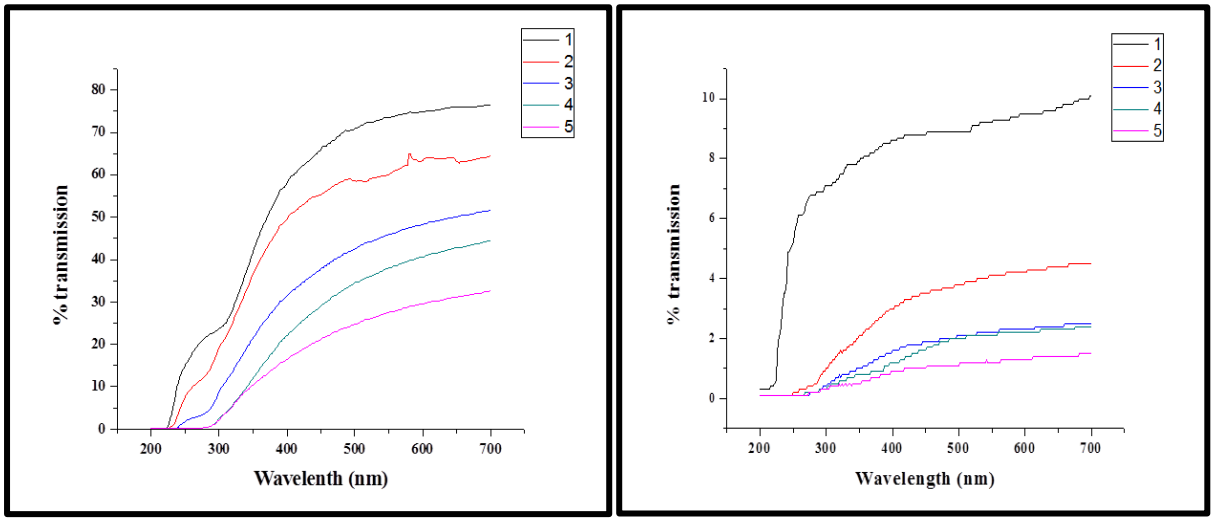
[Sample1-Chitosan(C)/Alginate(A),Sample2-E-C(1:4)/E-A(1:4),Sample3-E-C(2:3)/EA(2:3),Sample4-E-C(3:2)/E-A(3:2), Sample 5-E-C(4:1)/E-A(4:1)]

After comparing the thickness between the alginate-ECM composite films & chitosan-ECM composite films- it was observed that the thickness of Alginate-ECM composite films is more than

the thickness of Chitosan-ECM composite films. This may be because of higher amount of NaOH solubilized ECM (approx. 100mg) in alginate films than acetic acid solubilized ECMin case of chitosan films. Further it was observed than the Acetic Acid solubilized ECM turn into crystalline pattern leads to more compactness than the alginate-composite films. Otherwise, the only alginate films without ECM are of lower thickness than only Chitosan Film without ECM.

4.2 Film Transparency/ Film Light Transmission Test:

Normally %transmission of chitosan films is more than the alginate films but in all the films addition of the ECM leads to more opacity or reducedtransparency. This is obvious as ECM by itself is an opaque product.



(a) **(b)**

Fig 3: Film Light Transmission test of Chitosan-ECM Film (a) Alginate- ECM Film (b)
[1-Chitosan(C)/Alginate(A),2-E-C(1:4)/EA(1:4),3-E-C(2:3)/E-A(2:3),4-E-C(3:2)/E-A(3:2),5-E-C(4:1)/E-A(4:1)]

4.3 Swelling Test:

In the swelling test it was observed that with increase in the proportion of ECM. The % swelling also increases. This might be because of more hydrophilicity of ECM in comparison in to the polymers.

Film 1											
	0	15 mint	%swelling	30 mint	%swelling	60m int	%swelling	90 mint	%swelling	120 mint	%swelling
i	4	8	1	10	1.5	12	2	14	2.5	15	2.75
ii	4	9	1.25	11	1.75	12	2	13	2.25	15	2.75
iii	3	7	1.33	10	2.33	11	2.66	12	3	14	3.66
Film 2											
ii	5	15	2	17	2.4	19	2.8	21	3.2	24	3.8
ii	5	14	1.8	16	2.2	18	2.6	21	3.2	25	4
iii	5	11	1.2	18	2.6	18	2.6	22	3.4	24	3.8
Film 3											
i	5	10	1	12	1.4	13	1.6	15	2	17	2.4
ii	6	11	0.83	14	1.33	16	1.66	17	1.83	19	2.16
iii	5	9	0.8	13	1.6	14	1.8	17	2.4	19	2.8
Film 4											
i	5	7	0.4	12	1.4	14	1.8	15	2	17	2.4
ii	4	7	0.75	12	2	13	2.25	15	2.75	16	3
iii	6	9	0.5	14	1.33	15	1.5	17	1.83	19	2.16
Film 5											
i	3	6	1	9	2	10	2.33	12	3	14	3.66
ii	4	7	0.75	10	1.5	11	1.75	14	2.5	16	3
iii	3	6	1	9	2	10	2.33	14	3.66	15	4

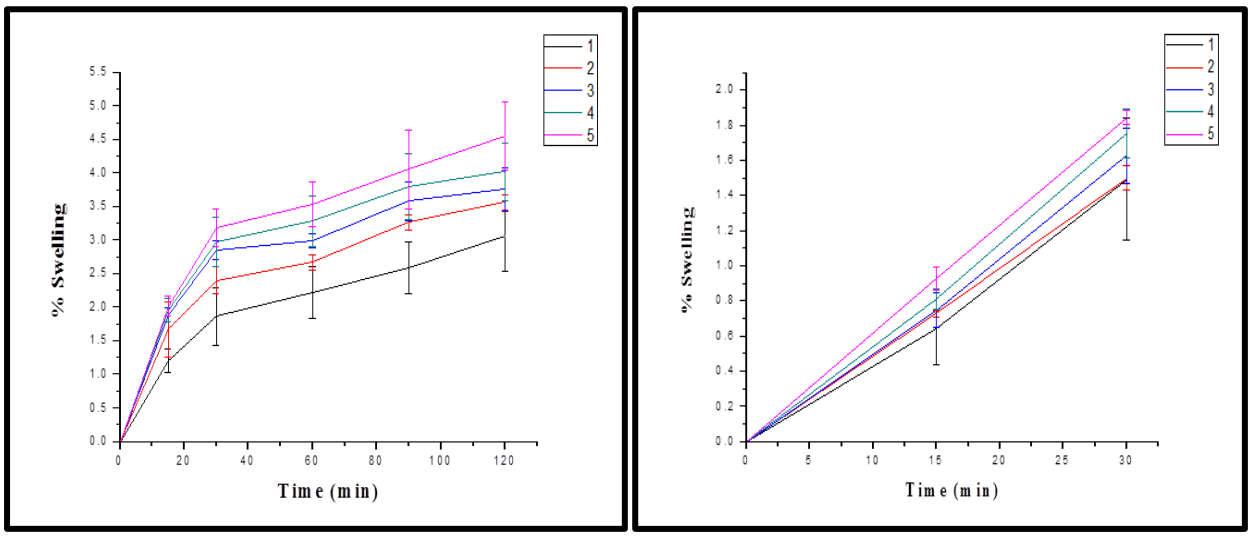
Table 5: Swelling Test result ofChitosan- ECM Film

Time (mint)	FILM 1	FILM 2	FILM 3	FILM 4	FILM 5
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
0	0±0	0±0	0±0	0±0	0±0
15	1.1944±0.1735	1.6667±0.4163	1.8778±0.1072	1.955±0.1803	2.0167±0.1443
30	1.8611±0.4276	2.4±0.2	2.8444±0.1388	2.9778±0.3672	3.1833±0.2887
60	2.2222 ± 0.3849	2.6667 ±0.1155	2.9889±0.1018	3.285±0.3775	3.5389±0.3368
90	2.5833 ± 0.3819	3.2667±0.1155	3.5778±0.2912	3.7944±0.4883	4.0556±0.5853
120	3.0556 ± 0.5292	3.5667±0.1155	3.7556±0.3203	4.0222±0.4299	4.5556±0.5092

Table 6:Swelling Test result of Chitosan- ECM Film

Film 1							
	0	15 mint	%swelling	Mean±S.D.	30 mint	%swelling	Mean±S.D.
i	6	10	0.66		15	1.5	1.49±0.345
ii	7	10	0.42		15	1.14	
iii	6	11	0.83	0.64±0.207	17	1.83	
Film 2							
ii	7	12	0.71		18	1.57	
ii	8	14	0.75		20	1.5	
iii	7	12	0.71	0.72±0.021	17	1.42	1.5±0.071
Film 3							
i	7	12	0.71		19	1.71	
ii	7	13	0.85		19	1.71	
iii	9	15	0.66	0.74±0.094	22	1.44	1.624±0.155
Film 4							
i	9	16	0.77		21	1.33	
ii	8	15	0.87		20	1.5	
iii	9	16	0.77	0.81±0.056	20	1.22	1.351±0.139
Film5							
i	9	17	0.88		24	1.66	
ii	9	17	0.88		24	1.66	
iii	10	20	1	0.92±0.064	26	1.6	1.6±0.038

Table 7: Swelling Test result ofAlginate- ECM Film



(a) **(b)**

Fig 4: SwellingChitosan-ECM Film (a) Alginate- ECM Film (b)

[1-Chitosan(C)/Alginate(A),2-E-C(1:4)/E-A(1:4),3-E-C(2:3)/E-A(2:3),4-E-C(3:2)/E-A(3:2),5-E-C(4:1)/E-A(4:1)]

Generally the % swelling of chitosan films are more than alginate films. Another interesting finding in the alginate films lasts less than the chitosan films in PBS before getting disintegrated. This may be because of the uncross linking nature of the films. So increase ECM concentration will lead to more retention of water in the films. Hence will be more useful for the dry wound for which the moisture or water is necessary for better wound healing.

Alginate films were found to disintegrate within 30 mints whereas Chitosan films were found to not disintegrate even after 2 hours. This may be because of more ECM in the Chitosan films which within themselves or with Chitosan films some short of bonds. Whereas in Alginate-ECM chitosan films might not have participated in any type of bond formation.

4.4 Moisture Test:

Moisture tests are due to check the amount of moisture that any materials absorb when they were exposed to environmental condition. Moisture test of the ECM-Chitosan & ECM-Alginate films was done. In our study we chose 84% of humidity and 30°C temperature keeping in view the atmosphere of India. The 84% humidity was created by supersaturated solution of KCl inside the desiccator and the desiccator was kept outside environment which on average was 25°C.

As it was mentioned earlier that the ECM is more hydrophilic than alginate & chitosan. So addition ECM should lead to more moisture absorption. On results also corroborating with the hypothesis.

FILM	Weight (Initial)	Weight (Final)	%moister absorption
1	7	9	28.571
2	11	16	45.454
3	7	11	57.142
4	8	13	62.500
5	10	17	70

Table 8:Moisture test result of Chitosan- ECM Film

FILM	Weight (Initial)	Weight (Final)	%moister absorption
1	15	22	46.667
2	23	36	56.521
3	27	47	74.074
4	17	37	117.647
5	15	38	153.33

Table 9:Moisture test result of Alginate- ECM Film

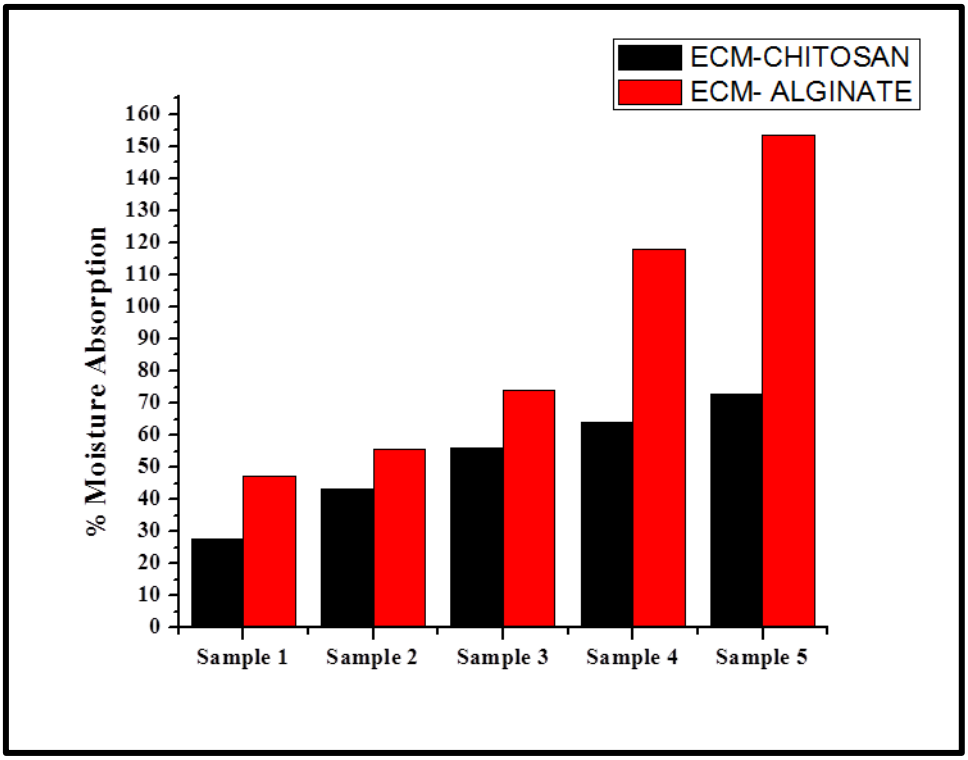


Fig 5: Moisture absorption test of Chitosan-ECM Film & Alginate- ECM Film

[Sample1-Chitosan(C)/Alginate(A),Sample2-EC(1:4)/EA(1:4),Sample3-EC(2:3)/EA(2:3),Sample4 EC(3:2)/EA(3:2),Sample 5-EC(4:1)/EA(4:1)]

4.5 Hemocompatibility Test:

In the hemocompatibility test it was observed that hemocompatibility of Alginate-ECM composite films are better than Chitosan-ECM composite Films. In this study we saw that % hemolysis of all ECM-Polymer composite films are less than 5. So these are highly hemocompatible. Other case

when I use only Chitosan & Alginate than its % hemolysis is greater than 5. So these are less hemocompatible.

FILM	O.D.	% Hemolysis
1	0.054	5.72467
2	0.058	4.8
3	0.057	4.60633
4	0.053	4.19567
5	0.038	3.75233

(a)

FILM	O.D.	% Hemolysis
1	0.057	5.35267
2	0.078	4.56967
3	0.074	4.28633
4	0.044	3.59833
5	0.063	3.319

(b)

Positive Control	1.71
Negative Control	0.13

(c)

Table 10:Hemocompatibility test result of ECM-Chitosan Film (a) & ECM Alginate Film (b)

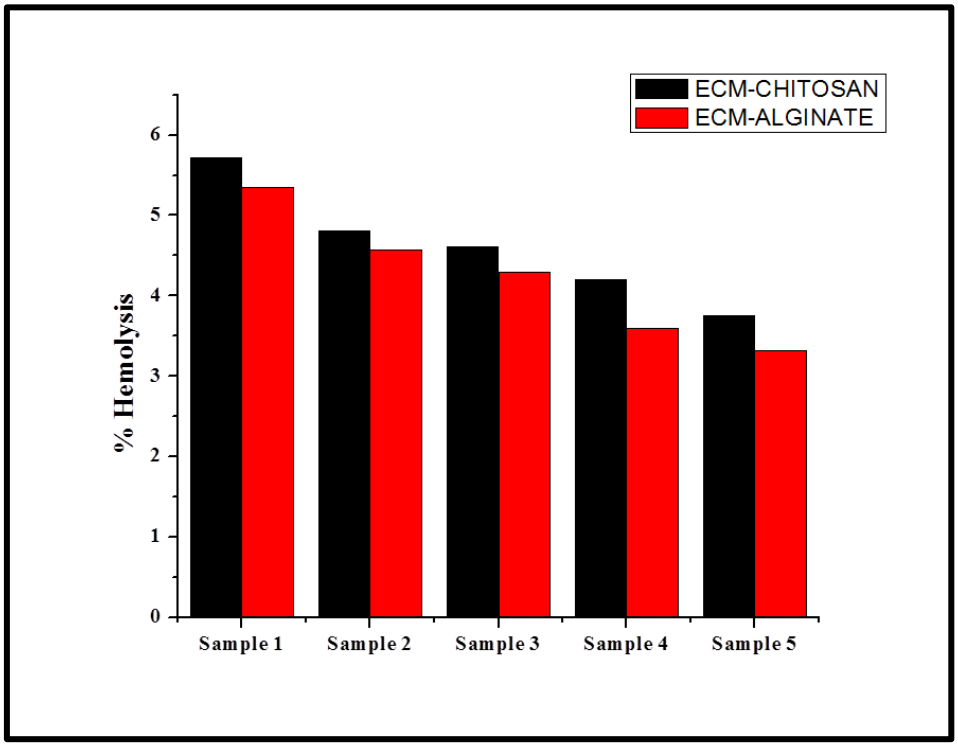


Fig 6: Hemocompatibility test of ECM-Chitosan Film& ECM Alginate Film

[Sample1-Chitosan(C)/Alginate(A),Sample2-EC(1:4)/EA(1:4),Sample3-EC(2:3)/EA(2:3),Sample4-EC(3:2)/EA(3:2),Sample 5-EC(4:1)/EA(4:1)]

4.6 Water Vapor Permeability Test:

Comparing between Chitosan-ECM composite Films and Alginate-ECM composite Films, Alginate Films are generally more permeable than Chitosan Films.

Film	0	6hrs	24 hrs	2 days	3days	4days	5days	6days	wvtr
1	44.279g	44.092g	43.42g	42.96g	41.962g	41.532g	40.964g	40.264g	4.16
2	40.825g	40.703g	40.15g	39.78g	39.132g	38.921g	38.264g	37.756g	3.54833
3	41.114g	40.996g	40.43g	40.06g	39.410g	38.586g	38.025g	37.964g	3.704
4	43.510g	43.343g	42.67g	42.30g	41.502g	40.903g	39.229g	39.883g	3.51233
5	40.562g	40.434g	39.89g	39.55g	38.85g	38.523g	37.903g	37.505g	3.29667

Table 11: Water Vapor Permeability Testresult of Chitosan- ECM Film

Film	0	6hrs	24 hrs	2 days	3days	4days	5days	6days	wvtr
1	45.398g	45.065g	44.596g	43.963g	42.993g	42.421g	41.852g	41.096g	4.7
2	42.705g	42.207g	41.993g	41.374g	40.871g	40.285g	39.912g	39.227g	4.33333
3	43.410g	43.319g	43.012g	42.769g	42.388g	41.758g	41.029g	40.632g	3.882
4	42.293g	41.964g	41.231g	40.586g	40.167g	39.852g	39.211g	38.953g	3.7124
5	44.283g	43.834g	43.125g	42.732g	42.732g	42.151g	41.432g	39.980g	3.49667

Table 12: Water Vapor Permeability Testresult of Alginate- ECM Film

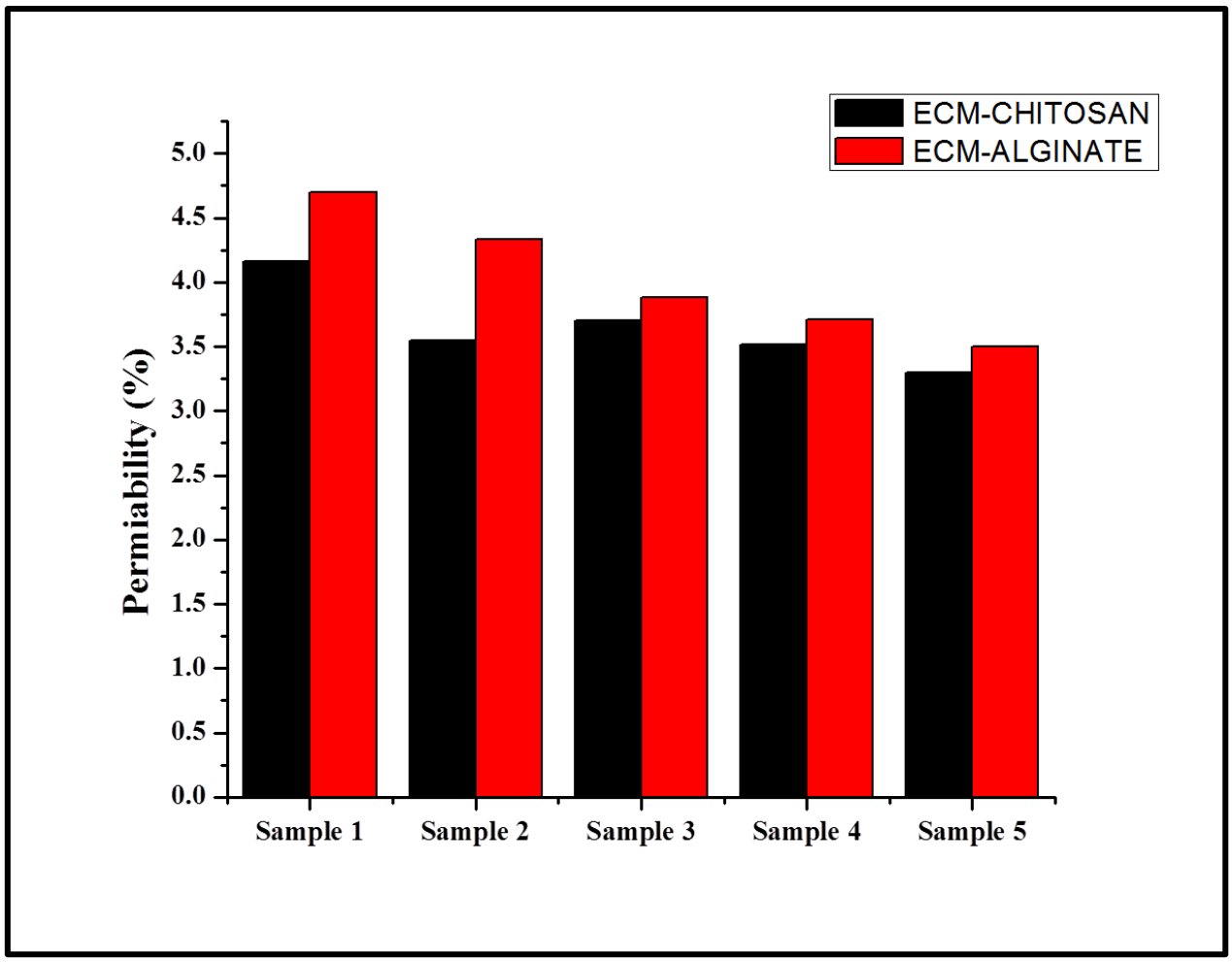
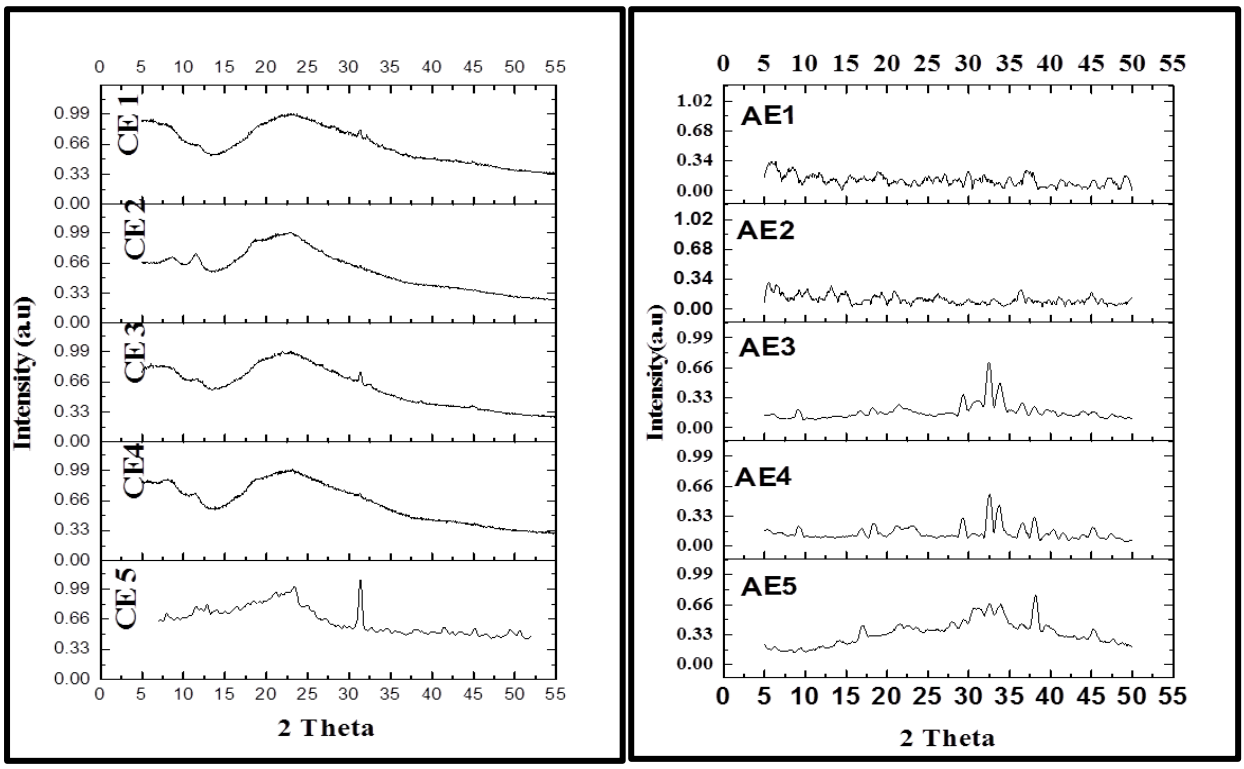


Fig 7: Water Vapor Permeability TestofChitosan-ECM Film &Alginate- ECM Film

[Sample1-Chitosan(C)/Alginate(A),Sample2-EC(1:4)/EA(1:4),Sample3-EC(2:3)/EA(2:3),Sample4-EC(3:2)/EA(3:2),Sample 5-EC(4:1)/EA(4:1)]

4.7 X-Ray Diffraction

Fig 8 shows that X-Ray Diffraction of ECM-Chitosan Film (a) & ECM-Alginate Film (b)



(a) **(b)**

Fig 8: X –Ray Diffraction Pattern of Chitosan-ECM Film (a) Alginate- ECM Film (b)

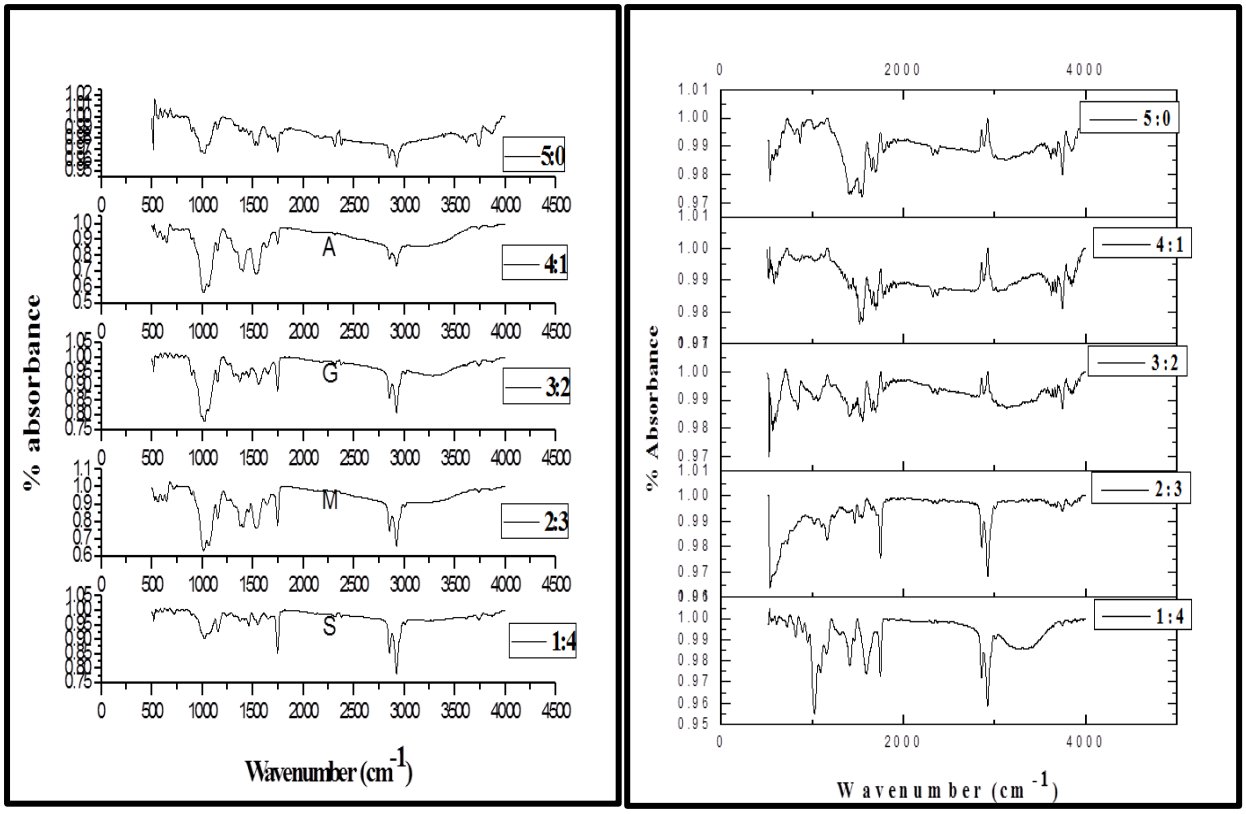
The X-ray diffraction pattern of Chitosan-ECM and Alginate-ECM demonstrated the characteristic peaks near 10° and 20° corresponding to pure chitosanand at 13.5°, 25°, and 39° corresponding to pure alginate. It is important to mention that the chitosan – ECM film showed characteristic peak at 15° and 23° while alginate-ECM peaks were preset at 10°, 21°, 32.3°, 35° and 39°. Lesser the polymer & higher the ECM composition crystallinity of the films increases. Drying may be leading to more crystallinity of ECM.

4.8 Fourier Transform Infrared Spectroscopy

The FTIR spectrum of chitosan-ECM & alginate-ECM composite films showed their characteristic peaks of absorption as demonstrated in Table 13. From the analysis it is confirmed that absorption peaks were observed at 3439 cm^{-1} which is N-H stretching bonds. C=O functional group is observed in 1666 cm^{-1}. 1438 cm^{-1}. Other peak is observed in 1363 cm^{-1} which is CH_3 bending vibration. 1155 cm^{-1} which is C–O–C functional group. There is a slight peak shift observed in chitosan sample which indicates interaction of ECM with chitosan.

	Functional Groups	**Wave number (cm^{-1})**
Chitosan	CH_3	1363
	C–O–C	1155
	C–N	1438
	C=O	1666
	N–H	3439
	O–H	3251
Alginate	COO-	1407
	OH−	3242
	C-O-C	1081 to 1024
	carboxyl &carboxylate	1000to1400
ECM	N-H	1500-1585
	C=O, C–N	1585-1720
	CH_3 , CH_2 , COO^-	1300-1500
	C-O & C-OH	985-1140

Table 13: Wave number of different functional groups in Chitosan, Sodium Alginate and ECM



(a) **(b)**

Fig 9: Fourier Transform Infrared Spectroscopy of Chitosan-ECM Film (a) Alginate- ECM Film (b)

In a similar context, the Alginate-ECM composite films were found to demonstrate the characteristic absorption peaks at3242 cm^{-1}, 1407 cm^{-1} , 1081 to 1024 cm^{-1} , and 1000 to 1400 cm−1. It is important to mention that both the composite films had characteristic peaks at 1630 cm^{-1}, 1632 cm^{-1}, 1638 cm^{-1}, and 1644 cm^{-1} which are belongs to Amide I bands; 1567 cm^{-1}, 1585 cm^{-1}, and 1599 cm^{-1} which is belongs to Amide II bands; 1243 cm^{-1} which is belongs to Amide III bands. There are new peaks such 3332 cm^{-1},3632 cm^{-1} which represent the interaction between two component of composite.

Chapter 5

CONCLUSION AND FUTURE WORKS

Alginate films without ECM were found to be less thick than the chitosan films without ECM. Upon swelling the films differed in the hydrophilicity as uptake of water by chitosan films were more than alginate films. This accounts to the moisture retention capacity of films Apart from this since ECM is more hydrophilic than alginate and chitosan and addition of higher proportion of ECM in films imparts them more hydrophilicity. Another interesting point is that greater the proportion of ECM in the films, higher is the crystallinity. Further in-vitro and in-vivo characterizations will give more information regarding its utility.

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